Prenatal Diagnosis Using Fetal Genetic Material in Maternal Circulation

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SUMMARY

In this paper, we review the major highlights in the development of clinical noninvasive prenatal diagnostic approaches analyzing fetal genetic material recovered from maternal circulation. [*Taiwanese J Obstet Gynecol* 2005; 44(1):8–15]

Key Words: fetal cell, fetal DNA, maternal blood, noninvasive, prenatal diagnosis

Introduction

In the second half of the 20th century, the prenatal diagnosis armamentarium changed dramatically with the introduction of amniocentesis and chorionic villus sampling. Both modalities are, however, invasive techniques and carry a certain risk, albeit low (0.5–1%), of pregnancy loss [1]. Routine noninvasive methods of prenatal diagnosis include first- and second-trimester ultrasonography and maternal serum biochemical screening. When screening women less than 35 years of age, second-trimester measurements of α-fetoprotein, human chorionic gonadotropin (hCG), and estriol correctly identify only 60–70% of cases of aneuploidy, with a calculated false-positive rate up to 5% [2]. Prenatal ultrasound examination in the second and third trimesters allows detection of only 17% of fetal anatomic abnormalities [3]. In fact, in the USA, approximately 1 in 1,000 live-born fetuses are postnatally diagnosed with Down syndrome. Most (80%) of these infants are born to women under the age of 35 who have no indications for routine invasive prenatal diagnostic procedures [4].

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First-trimester ultrasonographic evaluation of fetal nuchal translucency improves the aneuploidy detection rate up to 82% but requires special sonographic expertise [5]. Recent studies demonstrate that addition of first-trimester biochemical markers (free β-hCG and pregnancy-associated plasma protein-A) can increase sensitivity and improve false-positive rates [6]. Other first-trimester sonographic markers, including the absence of nasal bone and abnormal ductus venosus flow, have potential to improve the positive predictive value of screening, but require further validation [7,8]. Neither of these new techniques are devoid of false-positive results and both are associated with high health care costs of invasive follow-up studies and patient emotional anxiety [4].

The recovery of fetal genetic material in the form of fetal cells or cell-free fetal DNA from maternal circulation has, therefore, tremendous potential for prenatal diagnosis. Analysis of fetal genetic material acquired by means of routine venipuncture can potentially replace current standard biochemical screening or become an additional noninvasive technique to reduce the false-positive rates of biochemical and ultrasonographic screening programs.

Fetal Cells in Maternal Circulation

Fetal trophoblasts in maternal blood were first observed by the German pathologist Schmorl in 1893 during postmortem examination of the lung tissue of women

dying from eclampsia [9]. These findings were subsequently confirmed by other authors, but the question remained of whether fetal cells are routinely present in the maternal blood of healthy women. In 1957, Kleihauer et al identified fetal cells in the peripheral blood of pregnant women using a stain for fetal hemoglobin [10]. Douglas et al, in 1959, sampled large uterine veins during cesarean sections in 13 non-preeclamptic patients and demonstrated that large multinucleated cells of fetal origin were present in eight women [11]. Clayton et al demonstrated progressive increase in the detection rate of fetal cells in maternal circulation as a function of gestational age [12]. Fetal cells were observed in 5% of pregnancies by 16 weeks of gestation, but this reached almost 50% by 40 weeks. In 1969, Walknowska et al performed karyotypical analysis of cultured lymphocytes derived from peripheral blood of pregnant women. In 19 of 22 women carrying male fetuses, 46XY cells were identified [13]. These results were effectively reproduced and, by the early 1970s, the concept of fetal cell transfer into maternal circulation had become widely accepted. The labor-intensive and low-yield cytogenetic methods used in the quoted studies were subsequently replaced by newly developed cell-sorting techniques.

Various fetal cells recovered from maternal circulation were evaluated to determine the ideal cell type for prenatal diagnosis. Trophoblasts, being uniquely fetal in origin and in constant close contact with the uterine stroma, initially appeared to be a realistic choice. Indeed, deportation of trophoblasts to maternal circulation occurs relatively early in the first trimester, and would be ideal for early prenatal diagnosis. More than 6,500 monoclonal antibodies specific for trophoblast antigens were developed to differentiate trophoblastic cells from maternal cells, but only a handful could be used for successful separation. Mueller et al used polymerase chain reaction (PCR) amplification of a Y-chromosome-specific sequence in isolated trophoblasts to correctly predict fetal gender in 12 of 13 evaluated pregnancies [14]. Significant cross-reactivity between apparently trophoblast-specific monoclonal antibodies and other cell types have been reported by Bruch et al, where "pure" monoclonal antibodies GB17, GB21, and GB25 failed to identify trophoblastic cells in maternal circulation [15]. Apart from the relative paucity of monoclonal antibodies, other factors make trophoblast cells unlikely candidates for prenatal diagnosis. On entering maternal blood, trophoblasts are rapidly cleared by the maternal pulmonary microcirculation and are, therefore, rarely found in peripheral blood. The 1% prevalence of confined placental mosaicism makes trophoblast sprouts an unreliable representation of the exact chromosomal complement of the fetus. Additionally, the multinucleated nature of trophoblast sprouts renders them unsuitable for analysis using fluorescent *in situ* hybridization (FISH), which requires visualization of individual nuclei.

Paternally inherited human leukocyte antigen (HLA) allows initial separation of fetal lymphocytes from maternal blood via flow cytometry [16]. This approach, however, requires paternal HLA typing and is not possible when parental HLA antigens are identical. To complicate matters further, fetal production of lymphocytes is not initiated until the second trimester, further delaying prenatal diagnosis based on fetal lymphocyte identification. Persistence of fetal lymphocytes in maternal circulation has been described to be as long as 27 years following the index pregnancy, and these findings can be misleading when isolation of fetal lymphocytes is performed in multigravid women, because separated cells might not necessarily represent the current pregnancy [17].

Among other fetal cells isolated in maternal circulation, fetal platelets and granulocytes hold little promise for successful prenatal diagnosis. Fetal platelets are devoid of nuclear genetic material and are generally not useful for karyotyping and amplification of nuclear genomic sequences [18]. The limited number of fetal granulocytes detected in maternal blood specimens impedes their use as target cells for noninvasive prenatal diagnosis. It is estimated that fetal granulocytes constitute only 0.02–0.16% of the mononuclear cells present in peripheral blood, and the role of these cells in prenatal diagnosis remains largely unexplored [19].

Fetal nucleated red blood cells currently appear to be the most realistic candidate for noninvasive prenatal diagnosis. Firstly, nucleated red blood cells are relatively rare in adult maternal circulation, except under conditions of expansion of erythroid precursors, but are quite abundant in the fetus, particularly during first-trimester yolk sac hematopoiesis. Secondly, the nucleus of the fetal red blood cell contains the full complement of genetic material, which can be subjected to standard genetic analysis once the cells are isolated. Thirdly, nucleated red blood cells represent a relatively well-differentiated cell type and are characterized by a short life span, making it unlikely that they will persist in maternal circulation from one pregnancy to another.

Nucleated fetal red blood cells possess several unique cell-surface markers that can be used for automated cell-sorting and enrichment. These markers include, among others, a specific transferrin receptor antigen (CD71), which is expressed only at the erythroblast stage of development of red blood cells, and glycophorin A, which is uniquely expressed by the erythroid cell line.

Together, these cell-surface antigens, when applied to isolation of fetal nucleated red blood cells, allow 94% accuracy in prediction of fetal gender, as reported by Wachtel et al [20]. The search for a truly specific monoclonal antibody unique to fetal red blood cell antigens is still unfinished.

Fetomaternal transfer of nucleated red blood cells has been reported to occur as early as 6 weeks of gestation [4]. The estimated ratio of fetal nucleated red blood cells to maternal cells has been quoted at 1:100,000 in the first trimester and 1:10,000 at term [21]. Enrichment of fetal nucleated erythrocytes from maternal peripheral blood relies on the difference in cell size, volume, and DNA content. Based on these parameters, fetal cells can initially be separated by density gradient centrifugation or lysis of maternal anuclear erythrocytes. The remaining cell population can be further purified using magnetic-activated cell sorting (MACS), fluorescence-activated cell sorting (FACS), antibody-conjugated columns, or immunomagnetic beads. A recent prospective, multicenter clinical project demonstrated the superiority of MACS over FACS in target-cell recovery and fetal-cell detection [22].

Genetic analysis of isolated nucleated fetal red blood cells is hindered by their resistance to mitogen action and is, therefore, limited to FISH or PCR methodologies. These techniques do not require the presence of a dividing cell, but neither of them is capable of providing complete karyotypic characteristics. PCR allows detection of known fetal gene sequences, but will generally not help if both parents are carriers of the same autosomal recessive mutation or if the disease is X-linked. FISH allows detection of chromosomal numerical abnormalities and fetal gender. Other inherent limitations of FISH stem from its inability to detect most structural chromosomal rearrangements, its lack of sensitivity to mosaicism, and unknown rates of cross-hybridization and standard background hybridization.

In 1990, Camaschella et al were the first to achieve successful prenatal diagnosis of an inherited disease using fetal cells recovered from maternal blood [23]. Fetal hemoglobin Lepore-Boston disease was diagnosed using PCR analysis of fetal nucleated red blood cells isolated from maternal blood. PCR primers were used to amplify a known mutation sequence of a hybrid delta-beta gene mapped to chromosome 11. Two affected pregnancies were detected and both were confirmed by chorionic villus sampling. Other unique fetal sequences were subsequently detected by PCR and included Y-chromosome genes, mutations associated with β-thalassemia and sickle-cell anemia, survival motor neuron gene mutations associated with spinal muscular atrophy, mutations associated with Duchenne muscular dystrophy, and HLA DR and DQ alpha genes [4,18,24].

In 1994, Lo et al reported successful use of PCR with nucleated fetal red blood cells enriched from maternal blood for detection of fetal Rhesus (Rh) genotype in three cases of RhD sensitized pregnancies [25]. Fetal nucleated red blood cells were recovered from second-trimester specimens of maternal blood and PCR amplification using RhD gene primers allowed correct prediction of the fetal rhesus status that was confirmed via cordocentesis. Geifman-Holtzman et al, using the same principle, demonstrated a 100% positive predictive value and 67% negative predictive value in fetal Rh typing in maternally-derived fetal nucleated red blood cells [26]. Similar results were reported by Sekizawa et al [27].

In 1991, Price at al were the first to report successful prenatal diagnosis of fetal numerical chromosomal abnormality using FISH cytogenetic analysis of maternally derived fetal cells [28]. Fetal cells enriched from maternal blood at 10 weeks' gestation demonstrated three fluorescent signals on FISH with a chromosome-18 probe. Suspected fetal trisomy 18 was confirmed by chorionic villus sampling and tissue karyotyping upon termination of pregnancy. The following year, Elias et al reported the first case of prenatally diagnosed trisomy 21 using FISH in maternally derived fetal cells [29]. The reproducibility of these results was confirmed and, from 1998, almost all numerical abnormalities of fetal autosomes and sex chromosomes have been detected in maternal blood by FISH [4].

The practicality of prenatal diagnosis with PCR and FISH in maternally derived fetal cells was recently assessed in a prospective, multicenter Fetal Cell Isolation Study conducted by the National Institute of Child Health and Human Development. The study involved nine different academic centers (8 in the USA and 1 in Switzerland), and represents the largest and most rigorous study performed in the field. The goal was to determine the cytogenetic accuracy of fetal gender and fetal aneuploidy detection in fetal cells derived from maternal blood, compared with amniocentesis and chorionic villus sampling. The results from the first 5 years of the study were recently reported [22]. A total of 2,948 blood samples were processed and demonstrated that the aneuploidy detection rate using fetal cell analysis from maternal blood (74.4%; 95% confidence interval, 76.0%, 99.0%) was comparable to that of single-marker prenatal serum screening. The second part of the study is incorporating adjustments to ensure uniformity in shipping, processing, and scoring protocols across centers.

Cell-free Fetal DNA in Maternal Circulation

A relatively new concept in the field of prenatal diagnosis based on fetal genetic material detected in the maternal circulation is analysis of cell-free fetal DNA present in maternal blood. In 1977, Leon et al demonstrated that large quantities of cell-free DNA are present in the serum of patients with neoplasms, while minute amounts of cell-free DNA can be recovered from serum of healthy individuals (mean, 13 ± 3 ng/mL) [30].

Following the same train of thought, Lo et al, in 1997, were the first to use PCR with *SRY* gene primers on plasma from women pregnant with male fetuses. These experiments recovered significant quantities of male fetal DNA sequences in maternal plasma and serum [31]. The same group later demonstrated that the concentration of cell-free fetal DNA is significantly higher in maternal plasma than serum and increases in both compartments as a function of gestational age [32]. Mean concentration of cell-free fetal DNA measured during the first and early second trimester in serum versus plasma was 0.13% versus 3.4 %, and increased later in pregnancy to 1% versus 6.2%.

The precise cellular origin of cell-free fetal DNA in maternal blood is currently unknown. The universality of the previously discussed fetomaternal cellular transport during pregnancy suggests that constantly transmigrating fetal cells and placental trophoblastic cells are continuously destroyed by the maternal immune system, liberating fetal DNA into the circulation. Alternatively, naturally occurring apoptosis of fetal cells entering maternal blood may be responsible for the release of fetal DNA [33]. The progressive increase in fetal DNA concentration in maternal plasma and serum may be facilitated by the developmental apoptotic process in placental tissue that has been reported to increase as pregnancy advances [34]. Active remodeling of trophoblasts results in deportation of liberated DNA into the maternal circulation, and may enhance the permeability of the fetomaternal interface, leading to higher numbers of fetal cells entering the maternal circulation. This notion is further supported by the increased fetomaternal transfusion in conditions associated with structural and functional pathology of the uteroplacental unit: pre-eclampsia, intrauterine growth restriction, and aneuploidy [35,36].

Circulating cell-free fetal DNA is short-lived compared with intact fetal cells in maternal blood. Lo et al studied postpartum clearance of fetal DNA and demonstrated that, in most cases, the levels were undetectable by conventional methods as early as 2 hours postpartum [37]. Such a short half-life associated with consistent detection of cell-free DNA throughout gestation makes this approach particularly attractive for noninvasive prenatal diagnosis, as identified fetal DNA sequences appear to be unique to the current pregnancy and are extremely unlikely to represent prior gestations. Complex enrichment procedures used in detection of fetal cells in maternal plasma are not necessary for recovery of cell-free fetal DNA. Cell-free fetal DNA is more abundant than DNA extracted from nucleated red blood cells in the same volume of blood, and is readily analyzed by PCR assay [31].

In 1998, Lo et al described a real-time quantitative PCR assay for analysis of cell-free fetal DNA in maternal blood [32]. Leung et al, evaluating 20 women with gestational age between 26 and 34 weeks, demonstrated significantly higher concentrations of cell-free fetal DNA in women with spontaneous preterm delivery compared with those in women without preterm labor complications [38]. Interestingly, successful tocolysis was associated with lower concentrations of cell-free fetal DNA, and the authors propose this approach as a potential diagnostic test to identify patients in true preterm labor.

In a study comparing 20 third-trimester women with pre-eclampsia with 20 healthy controls, Lo et al noted a fivefold increase in the median concentration of cell-free fetal DNA in the former group (381 vs 76 genome-equivalents/mL; $p < 0.001$) [39]. These findings further support the hypothesis that the apoptotic process in the pre-eclamptic placenta contributes to the presence of cell-free fetal DNA in maternal blood, either independently or by facilitation of fetal cell transfer to maternal circulation.

Abnormally high concentrations of fetal genomic DNA measured in fetal cells recovered from maternal blood in women with a trisomy 21 fetus prompted assessment of cell-free fetal DNA concentrations in maternal blood in pregnancies with fetal aneuploidy compared with chromosomally normal fetuses [40, 41]. In 1999, Lo et al reported significantly higher concentrations of cell-free fetal DNA in pregnancies complicated by fetuses with trisomy 21 [41]. Results reached statistical significance ($p = 0.026$), but considerable overlap between values reported in the groups carrying chromosomally normal fetuses and fetuses with trisomy lowered the positive and negative predictive values of the test. Recently, Wataganara et al described the first noninvasive method for prenatal diagnosis of trisomy 13 [42]. Comparing cell-free fetal DNA concentrations in serum samples from pregnancies with confirmed trisomy 13 and 18 with those from pregnancies with the normal fetal chromosomal complement, they demonstrated significantly higher concentrations in the trisomy 13 but not trisomy 18 group ($p = 0.016$). These

findings create the possibility of a noninvasive prenatal screening tool for detection of fetal trisomy 13, which currently cannot be inferred from accepted biochemical screening.

Qualitative assessment of cell-free fetal DNA in maternal blood allows detection of paternally inherited microsatellite polymorphisms and mutations associated with single-gene disorders [43]. Fetal gender determination by demonstration of *SRY* gene sequences in maternal blood seemed the most natural application of the concept. Costa et al analyzed 121 first-trimester pregnant women carrying 61 karyotypically confirmed male and 60 karyotypically confirmed female fetuses. Cell-free fetal DNA was extracted from maternal blood and *SRY* PCR analysis demonstrated complete concordance with fetal sex: 100% sensitivity and 100% specificity [44]. Thus, reliable, noninvasive first-trimester fetal sex determination has been achieved.

High concentrations of cell-free fetal DNA in maternal plasma allowed a remarkable breakthrough in noninvasive determination of fetal RhD genotype. In 1998, Lo et al reported results of real-time PCR detection of RhD sequences in the plasma of 57 RhD-negative pregnant women [45]. In the samples obtained during the first trimester, there were two false-negative results, but results of RhD PCR analysis of maternal plasma fetal cell-free DNA obtained during the second and third trimesters demonstrated complete concordance with results obtained from serologic testing of cord blood or genotyping of amniotic fluid. Follow-up studies confirmed the findings of Lo et al, demonstrating the high reliability of the method, particularly during the second and third trimesters [46,47]. Reliable results are achieved even when different regions of the RhD gene are used in PCR amplification.

Analysis of paternally inherited microsatellite polymorphisms in cell-free fetal DNA sequences recovered from maternal blood has been described for chromosomes 13, 18, and 21, as well as the paternally inherited X-chromosome [48,49]. Clinical applicability of maternal plasma DNA analysis has been further supported by successful prenatal diagnosis of a number of single-gene disorders. Amicucci et al described prenatal diagnosis of myotonic dystrophy by detection of paternally derived expanded alleles of the dystrophia myotonica protein kinase gene in fetal DNA recovered from maternal plasma samples [50]. Saito et al reported noninvasive prenatal diagnosis of achondroplasia by demonstrating a diagnostic G-to-A transition at position 1138 of the fibroblast growth factor receptor gene in a DNA sample extracted from maternal plasma [51]. Additional examples of paternal polymorphisms and mutations that might be detected through analysis of cell-free fetal DNA extracted from maternal plasma include, among others, such autosomal recessive disorders as cystic fibrosis and hemoglobinopathies, when father and mother are carriers of discordant mutations [52]. A unique application of the methodology for antenatal diagnosis of fragile X syndrome was recently reported by Wald and Morris [53]. *SRY* PCR of fetal cell-free DNA was used to detect fetal gender, with subsequent invasive testing of male fetuses known to be at higher risk of the serious form of this disease. This approach opens clinical frontiers for application of noninvasive prenatal diagnosis for a wide spectrum of sex-linked diseases.

Conclusion

The present status of prenatal diagnosis using fetal genetic material in maternal circulation is in transition from the laboratory to active clinical practice.

It has become generally accepted that FISH and PCR analysis of fetal cells recovered from maternal circulation allow detection of gender and aneuploidy as well as identification of paternally inherited unique gene sequences. In specific instances, when parents are carriers of different mutations responsible for autosomal-recessive diseases, prenatal characterization of fetal status with respect to the paternally derived mutation is also possible. Sensitivity of fetal aneuploidy detection, as confirmed by a recent multicenter study, is at least comparable to that of second-trimester serum marker screening. This approach is, however, limited by maternal genetic material contamination and low fetal cell numbers recovered from maternal blood by current cell separation techniques.

Quantitative and qualitative assessment of cell-free fetal DNA in maternal plasma and serum can be important in the selection of pregnancies at risk of fetal aneuploidy and complications such as pre-eclampsia and preterm labor. Similar to PCR analysis of fetal cells, this approach allows prenatal detection of fetal gender and paternally inherited single-gene diseases. The large amounts and short half-life of cell-free fetal DNA present in maternal plasma and the rapidity and reproducibility of assays make this method highly attractive for future clinical application in analysis of known gene sequences, but not for karyotypic studies requiring FISH.

The full potential of prenatal diagnosis based on genetic material recovered from maternal circulation remains to be evaluated and utilized. The future perfection of fetal-cell enrichment techniques and methods for recovery of cell-free fetal DNA will broaden the clinical availability and application of these ap-

proaches. Quantitative assessment of cell-free fetal DNA and FISH analysis of fetal cells can become complementary to first- and second-trimester noninvasive screening. The incidence of false-positive results in first- and second-trimester biochemical and ultrasound testing requiring invasive cytogenetic procedures can be dramatically reduced by addition of noninvasive screening based on fetal genetic material present in maternal circulation. Alternatively, fetal genetic material testing can be routinely incorporated as a standard component of prenatal diagnosis algorithms.

Qualitative assessment of cell-free fetal DNA and PCR analysis of fetal cells in maternal blood can encompass noninvasive prenatal diagnosis and screening of common single-gene disorders, fetal RhD antigen status, and detection of fetal gender. In fact, diagnostic and clinically accepted testing of cell-free fetal DNA for determination of fetal RhD antigen status is currently offered in Spain and Basel, and is available on an experimental basis in several commercial laboratories in the USA. Noninvasive determination of fetal RhD status combined with ultrasonographic assessment of mid-cerebral artery peak systolic velocity may, in the near future, revolutionize the management of pregnancies at risk for Rh isoimmunization. This approach can obviate the need for amniocentesis to investigate fetal anemia and fetal RhD antigen genotyping.

Apart from prenatal diagnosis, evaluation of fetal genetic material in maternal circulation can have additional ramifications. Relatively recent identification of fetal cell differentiation in the tissue of patients suffering from autoimmune and immune-mediated diseases may shed light on the putative pathogenesis of these dysfunctions and on reparative tissue processes [54,55]. Successful *in vitro* expansion of fetal cells recovered from maternal blood can be implemented in the field of therapeutic cloning and enhance existing fetal cell-based noninvasive diagnostic protocols by dramatically increasing the availability of these cells [56]. However, the ability to clonally expand recovered fetal cells *in vitro* has not yet been fully established [57]. Intriguing and controversial identification of fetal mRNA sequences and intact fetal cells in maternal plasma opens future research directions towards better understanding of normal and pathologic functioning of the fetomaternal unit [58–60].

References

1. Lamvu G, Kuller JA. Prenatal diagnosis using fetal cells from the maternal circulation. *Obstet Gynecol Surv* 1997;52:433–7.
2. Haddow, JE, Palomaki GE, Knight GJ, et al. Prenatal screening for Down's syndrome with use of maternal serum markers. *N Engl J Med* 1992;327:588–93.
3. Ewigman BG, Crane JP, Frigoletto FD, LeFevre ML, Bain RP, McNellis D. Effects of prenatal ultrasound screening on perinatal outcome. RADIUS study group. *N Engl J Med* 1993; 329:821–7.
4. Bianchi DW. Current knowledge about fetal cells in the maternal circulation. *J Perinat Med* 1998;26:175–85.
5. Snijders RJM, Noble P, Sebire N, Souka A, Nicolaides KH. UK multicenter project on assessment of risk of trisomy 21 by maternal age and fetal nuchal-translucency thickness at 10–14 weeks of gestation. Fetal Medicine Foundation First Trimester Screening Group. *Lancet* 1998;352:343–6.
6. Bindra R, Heath V, Liao A, Spencer K, Nicolaides KH. One-stop clinic for assessment of risk of trisomy 21 at 11–14 weeks: a prospective study of 15030 pregnancies. *Ultrasound Obstet Gynecol* 2002;20:219–25.
7. Cicero S, Curcio P, Papageorghiou A, Sonek J, Nicolaides KH. Absence of nasal bone in fetuses with trisomy 21 at 11–14 weeks of gestation: an observational study. *Lancet* 2001;358: 1665–7.
8. Mavrides E, Sairam S, Hollis B, Thilaganathan B. Screening for aneuploidy in the first trimester by assessment of blood flow in the ductus venosus. *Br J Obstet Gynaecol* 2002;109: 1015–9.
9. Schmorl G. *Pathologisch-anatomische undersuchungen uber puerperaleklampsie*. Leipzig: Vogel, 1893.
10. Kleihauer E, Braun H, Betke K. Demonstration von fetalem Hamaglobin in der Erythrocyten eines Blutausstrihes. *Klin Wochenschr* 1957;35:637–8.
11. Douglas GW, Thomas L, Carr M, Cullen NM, Morris R. Trophoblasts in the circulating blood during pregnancy. *Am J Obstet Gynecol* 1959;78:960–73.
12. Clayton EM, Feldhaus WD, Whitacre FE. Fetal erythrocytes in the maternal circulation of pregnant women. *Obstet Gynecol* 1964;23:915–9.
13. Walknowska J, Conte FA, Grumbach MM. Practical and theoretical implications of fetal-maternal lymphocyte transfer. *Lancet* 1969;i:1119–22.
14. Mueller UW, Hawes CS, Wright AE, et al. Isolation of fetal trophoblast cells from peripheral blood of pregnant women. *Lancet* 1990;336:197–200.
15. Bruch JF, Metezeau P, Garcia-Fonknechten N, et al. Trophoblast-like cells sorted from peripheral maternal blood using flow cytometry: a multiparametric study involving transmission electron microscopy and fetal DNA amplification. *Prenat Diagn* 1991;11:787–98.
16. Herzenberg LA, Bianchi DW, Schroder J, Cann HM, Iverson GM. Fetal cells in the blood of pregnant women: detection and enrichment by fluorescence-activated cell sorting. *Proc Natl Acad Sci USA* 1979;76:1453–5.
17. Bianchi DW, Zickwolf GK, Weil GJ, Sylvester S, DeMaria MA. Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum. *Proc Natl Acad Sci USA* 1996;93: 705–8.
18. Geifman-Holtzman O, Blatman RN, Bianchi D W. Prenatal genetic diagnosis by isolation and analysis of fetal cells circulating in maternal blood. *Semin Perinatol* 1994;18:366–75.
19. Zilliacus R, De la Chapelle A, Schroder J, Tilikainen A, Kohne

E, Kleihauer E. Transplacental passage of foetal blood cells. *Scand J Haematol* 1975;15:333–8.
20. Wachtel S, Elias S, Price J, et al. Fetal cells in the maternal circulation: isolation by multiparameter flow cytometry and confirmation by polymerase chain reaction. *Hum Reprod* 1991;6:1466–9.
21. Hamada H, Arinami T, Kubo T, Hamaguchi H, Iwasaki H. Fetal nucleated cells in maternal peripheral blood: frequency and relationship to gestational age. *Hum Genet* 1993;91: 427–32.
22. Bianchi DW, Simpson JL, Jackson LG, et al. Fetal gender and aneuploidy detection using fetal cells in maternal blood: analysis of NIFTY I data. National Institute of Child Health and Development Fetal Cell Isolation Study. *Prenat Diagn* 2002;22:609–15.
23. Camaschella C, Alfarano A, Gottardi E, et al. Prenatal diagnosis of fetal hemoglobin Lepore-Boston disease on maternal peripheral blood. *Blood* 1990;75:2102–6.
24. Beroud C, Karliova M, Bonnefont JP, et al. Prenatal diagnosis of spinal muscular atrophy by genetic analysis of circulating fetal cells. *Lancet* 2003;361:1013–4.
25. Lo YM, Bowell PJ, Selinger M, et al. Prenatal determination of fetal rhesus D status by DNA amplification of peripheral blood of rhesus-negative mothers. *Ann NY Acad Sci* 1994;731: 229–36.
26. Geifman-Holtzman O, Bernstein IM, Berry SM, et al. Fetal RhD genotyping in fetal cells flow sorted from maternal blood. *Am J Obstet Gynecol* 1996;174:818–22.
27. Sekizawa A, Watanabe A, Kimura T, Saito H, Yanaihara T, Sato T. Prenatal diagnosis of the fetal RhD blood type using a single fetal nucleated erythrocyte from maternal blood. *Obstet Gynecol* 1996;87:501–5.
28. Price JO, Elias S, Wachtel SS, et al. Prenatal diagnosis with fetal cells isolated from maternal blood by multiparameter flow cytometry. *Am J Obstet Gynecol* 1991;165:1731–7.
29. Elias S, Price J, Dockter M, et al. First trimester prenatal diagnosis of trisomy 21 in fetal cells from maternal blood. *Lancet* 1992;340:1033.
30. Leon SA, Shapiro B, Sklaroff DM, Yaros MJ. Free DNA in serum of cancer patients and the effect of therapy. *Cancer Res* 1977;37:646–50.
31. Lo YMD, Corbetta N, Chamberlain PF, et al. Presence of fetal DNA in maternal plasma and serum. *Lancet* 1997;350:485–7.
32. Lo YMD, Tein MSC, Lau TK, et al. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. *Am J Hum Genet* 1998;62: 768–75.
33. Van Wijk IJ, De Hoon AC, Jurhawan R, et al. Detection of apoptotic fetal cells in plasma of pregnant women. *Clin Chem* 2000;46:729–31.
34. Smith S, Baker PN, Symonds EM. Placental apoptosis in normal human pregnancy. *Am J Obstet Gynecol* 1997;177:57–65.
35. Holzgreve W, Ghezzi F, Di Naro E, Ganshirt D, Maymon E, Hahn S. Disturbed fetomaternal cell traffic in preeclampsia. *Obstet Gynecol* 1998;91:669–72.
36. Zhong XY, Burk MR, Troeger C, Jackson LR, Holzgreve W, Hahn S. Fetal DNA in maternal plasma is elevated in pregnancies with aneuploid fetuses. *Prenat Diagn* 2000;20: 795–8.
37. Lo YMD, Zhang J, Leung TN, Lau TK, Chang AMZ, Hjelm NM. Rapid clearance of fetal DNA from maternal plasma. *Am J Hum Genet* 1999;64:218–24.
38. Leung TN, Zhang J, Lau TK, Hjelm NM, Lo YMD. Maternal plasma fetal DNA as a marker for preterm labour. *Lancet* 1998;352:1904–5.
39. Lo YMD, Leung TN, Tein MSC, et al. Quantitative abnormalities of fetal DNA in maternal serum in preeclampsia. *Clin Chem* 1999;45:184–8.
40. Bianchi DW, Williams JM, Sullivan LM, Hanson FW, Klinger KW, Shuber AP. PCR quantitation of fetal cells in maternal blood in normal and aneuploid pregnancies. *Am J Hum Genet* 1997;61:822–9.
41. Lo YMD, Lau TK, Zhang J, et al. Increased fetal DNA concentration in the plasma of pregnant women carrying fetuses with trisomy 21. *Clin Chem* 1999;45:1747–51.
42. Wataganara T, LeShane ES, Farina A, et al. Maternal serum cell-free DNA levels are increased in cases of trisomy 13 but not trisomy 18. *Hum Genet* 2003;111:204–8.
43. Lo YMD. Fetal DNA in maternal plasma: biology and diagnostic applications. *Clin Chem* 2000;46:1903–6.
44. Costa JM, Benachi A, Gautier E, Jouannic JM, Ernault P, Dumez Y. First-trimester fetal sex determination in maternal serum using real-time PCR. *Prenat Diagn* 2001;21:1070–4.
45. Lo YMD, Hjelm NM, Fidler C, et al. Prenatal diagnosis of fetal RhD status by molecular analysis of maternal plasma. *N Engl J Med* 1998;339:1734–8.
46. Faas BHW, Beuling EA, Christiaens GC, Von dem Borne AE, Van der Schoot CE. Detection of fetal RHD-specific sequences in maternal plasma. *Lancet* 1998;352:1196.
47. Bischoff FZ, Nguyen DD, Marquez-Do D, Moise KJ Jr, Simpson JL, Elias S. Noninvasive determination of fetal RhD status using fetal DNA in maternal serum and PCR. *J Soc Gynecol Investig* 1999;6:64–9.
48. Pertl B, Sekizawa A, Samura O, Orescovic I, Rahaim PT, Bianchi DW. Detection of male and female fetal DNA in maternal plasma by multiplex fluorescent polymerase chain reaction amplification of short tandem repeats. *Hum Genet* 2000;106:45–9.
49. Tang NLS, Leung TN, Zhang J, Lau TK, Lo YMD. Detection of fetal-derived paternally inherited X-chromosome polymorphisms in maternal plasma. *Clin Chem* 1999;45: 2033–5.
50. Amicucci P, Gennarelli M, Novelli G, Dallapiccola B. Prenatal diagnosis of myotonic dystrophy using fetal DNA obtained from maternal plasma. *Clin Chem* 2000;46:301–2.
51. Saito H, Sekizawa A, Morimoto T, Suzuki M, Yanaihara T. Prenatal DNA diagnosis of a single-gene disorder from maternal plasma. *Lancet* 2000;356:1170.
52. Pertl B, Bianchi DW. Fetal DNA in maternal plasma: emerging clinical applications. *Obstet Gynecol* 2001;98:483–90.
53. Wald NJ, Morris JK. A new approach to antenatal screening for fragile X syndrome. *Prenat Diagn* 2003;23:345–51.
54. Srivatsa B, Srivatsa S, Johnson KL, Samura O, Lee SL, Bianchi DW. Microchimerism of presumed fetal origin in thyroid specimens from women: a case-control study. *Lancet* 2001; 358:2034–8.
55. Artlett CM, Rasheed M, Russo-Stieglitz KE, Sawaya HH, Jimenez SA. Influence of prior pregnancies on disease course and cause of death in systemic sclerosis. *Ann Rheum Dis* 2002; 61:346–50.

56. Tutschek B, Reinhard J, Kogler G, Wernet P, Niederacher D. Clonal culture of fetal cells from maternal blood. *Lancet* 2000;356:1736–7.
57. Zimmermann B, Holzgreve W, Zhong XY, Hahn S. Inability to clonally expand fetal progenitors from maternal blood. *Fetal Diagn Ther* 2002;17:97–100.
58. Poon LLM, Leung TN, Lau TK, Lo YMD. Presence of fetal RNA in maternal plasma. *Clin Chem* 2000;46:1832–4.
59. Poon LLM, Leung TN, Lau TK, Lo YMD. Prenatal detection of fetal Down's syndrome from maternal plasma. *Lancet* 2000; 356:1819–20.
60. Bischoff FZ, Hahn S, Johnson KL, et al. Intact fetal cells in maternal plasma: are they really there? *Lancet* 2003;361: 139–40.